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Immunochemoradiotherapy for Patients with Oral Squamous Cell Carcinoma: Augmentation of OK-432–Induced Helper T Cell 1 Response by 5-FU and X-ray Irradiation[1]

Tomoyuki Tano*, Masato Okamoto†, Shin Kan‡, Takashi Bando§, Hiroyuki Goda*, Koh-ichi Nakashiro*, Shigetaka Shimodaira¶, Shigeo Koido#, Sadamu Homma, Tomonobu Fujita†, Mitsunobu Sato§, Naomi Yamashita††, Hiroyuki Hamakawa* and Yutaka Kawakami†**

*Department of Oral and Maxillofacial Surgery, Ehime University Graduate School of Medicine, Ehime, Japan; †Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan; ‡Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan; §Second Department of Oral and Maxillofacial Surgery, Tokushima University School of Dentistry, Tokushima, Japan; ¶Cell Processing Center, Shinshu University Hospital, Nagano, Japan; #Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan; **Department of Oncology, Institute of DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan; ††Laboratory of Pharmacotherapy, Research Institute of Pharmaceutical Sciences, Faculty of Pharmacy, Musashino University, Tokyo, Japan

Abstract

Eighty-one patients with oral squamous cell carcinoma (OSCC) received oral fluoropyrimidine UFT and radiotherapy (RT) with or without an immunotherapeutic agent OK-432. Both overall survival and progression-free survival of patients who received RT + UFT + OK-432 were significantly longer than those of patients who received RT + UFT ($P = .0075$ and $P = .0175$, respectively). Clinical response was also more favorable in RT + UFT + OK-432 group than in RT + UFT group ($P = .0066$). Next, *in vitro* experiments were conducted to examine the effect of 5-fluorouracil (5-FU) and X-ray irradiation in OK-432–induced immunity. Human peripheral blood mononuclear cells stimulated with OK-432 produced helper T cell 1 (Th1)–type cytokines as well as interleukin-10 (IL-10) and transforming growth factor–β (TGF-β), which are produced by Th2 and regulatory T cells (Tregs), respectively, and are inhibitory in antitumor immunity. OK-432–induced IL-10 and TGF-β but not Th1 cytokines were significantly inhibited by 5-FU and/or X-ray. 5-FU and X-ray also inhibited the expression of mRNAs for GATA-3 and Foxp3, which are transcription factors for Th2 and Tregs, respectively, but not for T-bet, a transcription factor for Th1. In addition, 5-FU and X-ray decreased the expression of mRNAs for suppressor of cytokine signaling 1 (SOCS1) and SOCS3. Antisense oligonucleotides for

Address all correspondence to: Dr Masato Okamoto, Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, 33 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. E-mail: mokamoto@a2.keio.jp

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SOCS1 and *SOCS3* markedly reduced OK-432–induced IL-10 and TGF-β. This is the first report clearly demonstrating that OK-432–based immunotherapy significantly enhanced the therapeutic effects of chemoradiotherapy in patients with OSCC as well as elucidating the mechanism of the synergistic effect of immunochemoradiotherapy in which 5-FU and radiation enhanced OK-432–induced Th1 response mediated by the inhibition of *SOCS1* and *SOCS3* gene expression.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) represents the fifth most frequently occurring cancer worldwide. Of the 1.6 million diagnoses and 333,000 deaths each year worldwide due to HNSCC, half are localized in the oral cavity [oral squamous cell carcinoma (OSCC)] [1]. Despite recent advances in surgery, radiotherapy (RT) and chemotherapy, the 5-year survival rate for patients with this disease has remained at 50% for the past 30 years [2]. In addition, functional or aesthetic disturbance by surgery for OSCC causes impairment of quality of life, and major surgery for patients of advanced age might be impossible due to compromised hosts. One approach to overcoming these problems is to develop a more effective therapeutic method for OSCC that reduces the disturbances induced by surgery.

UFT (Taiho Pharmaceutical Co, Tokyo, Japan) is an oral preparation combining tegafur, a prodrug of 5-fluorouracil (5-FU), and uracil in a 1:4 ratio that is used widely in Asian countries for treatment of solid tumors including HNSCC [3,4]. OK-432 (Chugai Pharmaceutical Co, Ltd, Tokyo, Japan), which is a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A), is being successfully used as an immunotherapeutic agent in many types of malignancies [5–7]. We have also reported that OK-432–based immunotherapy exhibits a marked therapeutic effect in patients with OSCC [8,9]. It has been reported that OK-432 elicits antitumor effects by stimulating immunocompetent cells such as macrophages, T cells, and natural killer cells [10,11] and that OK-432 induces interleukin-12 (IL-12) and polarizes the T cell response to a helper T cell 1 (Th1)–dominant state [12]. Recently, we and other investigators demonstrated that OK-432 induces maturation of dendritic cells (DCs), which are professional antigen-presenting cells, and that OK-432–stimulated DCs can induce tumor antigen–specific cytotoxic T lymphocytes *in vitro* as well as *in vivo* [13–16]. OK-432, which is generally called a "nonspecific immunotherapeutic agent," induces tumor antigen–specific immunity, and OK-432 is therefore being watched with great interest. Furthermore, we have clearly demonstrated the molecular events that an active component contained in the OK-432 augments antitumor host responses through Toll-like receptor (TLR)–related signals [15–19], which play essential roles in innate immune responses to microbial pathogens based on their ability to recognize pathogen-associated molecular patterns [20,21].

In the current study, we have investigated the efficacy of OK-432 in the antitumor effect of the chemoradiotherapy (CRT) in patients with OSCC and then examined *in vitro* the possibility that RT and UFT might augment OK-432–induced antitumor immunity. We elucidate the mechanism of the synergistic effect of the combination therapy and propose the concept of a new cancer therapy, an immunochemoradiotherapy (ICRT), based on the results of the current study.

Materials and Methods

Patients and Treatment Protocol

This study was carried out in accordance with the standards of our Institutional Committee for the Protection of Human Subjects. Informed written consent for study participation was obtained from all patients, and the collection of samples was approved by the Institutional Review Board. From 1994 to 2006, 81 patients (46 males and 35 females) with OSCC were treated with RT and UFT with or without OK-432 at the Second Department of Oral and Maxillofacial Surgery, Tokushima University Hospital. A summary of the patients' profiles appears in Table 1. The median patient age was 63.6 years (range, 36–85 years). On the basis of the Tumor-Node-Metastasis system for the classification of malignant disease [22], there were 7 T1 cancers, 48 T2 cancers, 20 T3 cancers, 6 T4 cancers, 41 N0 cancers, 28 N1 cancers, 11 N2 cancers, and 1 N3 cancer. All cancers were M0. All of the patients were histopathologically diagnosed as having SCC. Primary sites of the tumors were the tongue (n = 42), upper gingiva (n = 12), lower gingiva (n = 11), buccal mucosa (n = 6), floor of the mouth (n = 7), and hard palate (n = 3). All of the patients had primary diseases and had not received any previous treatment. None of the patients was severely immunodeficient as determined from clinical examinations.

All patients received UFT (300 mg/day for 8 weeks) simultaneously in combination with RT (a total irradiation dose of 50–60 Gy). Sixty-seven of the 81 patients were administered OK-432 intradermally or peritumorally at a dose of 0.5 Klinische Einheit, i.e., 50 μg/week. When any severe adverse events were not shown, the dose was increased up to 5 Klinische Einheit per week. Patients were monitored for general symptoms related to the therapy. Four weeks after completion of the therapy, biopsy materials were taken from all of the treated patients for the assessment of therapeutic effect. When any residual tumor cells were detected in biopsy material(s), surgical resection of the tumor(s) was performed. Of the patients who did not show complete response (CR), residual tumors in seven patients could not be surgically resected because of the patients' poor general condition, and one patient refused the operation. These eight patients received further chemotherapy. The tumor responses to the combination therapy were classified according to the Response Evaluation Criteria in Solid Tumors criteria. Adverse events were evaluated by grading toxicity according to the National Cancer Institute Common Terminology Criteria for Adverse Events guidelines, version 3.0.

Treatment In Vitro *of Human Peripheral Blood Mononuclear Cells*

Healthy volunteer-derived peripheral blood mononuclear cells (PBMCs; 1×10^7/ml) were isolated from heparinized venous blood by Ficoll-Hypaque gradient density centrifugation. The PBMCs were cultured in RPMI 1640 medium (Life Technologies, Inc, Gaithersburg,

Table 1. Summary of Characteristics of Patients with OSCC.

	RT + UFT + OK-432	RT + UFT	Total	*P* value (RT + UFT + OK-432 *vs* RT + UFT)
Number of patients	67	14	81	
Age	63.2 (range, 36–77)	65.1 (range, 38–85)	63.6 (range, 36–85)	.891
Sex				
M	38	8	46	1
F	29	6	35	
Stage				
I	5	0	5	.522
II	20	5	25	
III	28	8	36	
IV	14	1	15	
T				
1	7	0	7	.231
2	40	8	46	
3	14	6	22	
4	6	0	6	
N				
0	32	9	41	.715
1	24	4	28	
2	10	1	11	
3	1	0	1	
M				
0	67	14	81	1
1	0	0	0	
Primary sites				
Tongue	35	7	42	.768
Upper gingival	11	1	12	
Lower gingival	8	3	11	
Buccal mucosa	5	1	6	
Floor of the mouth	6	1	7	
Hard palate	2	1	3	

MD) containing 10% heat-inactivated FBS (Bio-Whittaker, Walkersville, MD), treated once with a single dose of 2 Gy X-rays *in vitro* using an X-ray irradiator (Hitachi Medico, Tokyo, Japan) and stimulated with OK-432 (1 μg/ml) and 5-FU (0.5 μg/ml; Kyowa Hakko, Tokyo, Japan) for 24 hours. In some experiments, PBMCs were irradiated (2 Gy) or were treated with 5-FU (0.5 μg/ml) 0, 3, 6, or 12 hours before OK-432 stimulation. To examine the role of suppressor of cytokine signaling 1 (SOCS1) and SOCS3, we added sense and antisense oligonucleotides (ODNs; 10 μM) of these molecules to the PBMC culture treated with OK-432 using Effectene Transfection Reagent (Qiagen, Inc, Valencia, CA). Sequences of sense and antisense ODNs were as given follows. *SOCS1* antisense, CACCTGGTTGTGTGCTACCATCCTAC; *SOCS1* sense, GTAGGATGGTAGCACACAACCAGGTG; *SOCS3* antisense, CGGGAAACTTGCTGTGGGTGACCAT; *SOCS3* sense, ATGGTCACCCACAGCAAGTTTCCCG. Supernatants were analyzed for cytokines by ELISA, and expression of mRNAs in the PBMCs was assayed by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) as described below.

Cytokine Assay

Cytokines in the supernatants were analyzed by using ELISA kits. The ELISA kits for human interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), transforming growth factor–β (TGF-β), IL-10, and IL-12 were purchased from BioSource International, Inc (Camarillo, CA), and the ELISA system for human IL-18 was purchased from MBL (Nagoya, Japan).

RNA Extraction and Semiquantitative RT-PCR

Expression of mRNAs for T-bet, GATA-3, Foxp3, SOCS1, SOCS3, and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), a housekeeping gene that was used as an internal control, was detected by semiquantitative RT-PCR. Total RNAs were extracted from the PBMCs by a modified acid guanidinium thiocyanate–phenol–chloroform using ISOGEN RNA extracting mixture (Nippon Gene, Toyama, Japan) according to the manufacturer's recommendations. One microgram of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc) and random primers (Life Technologies, Inc) in a volume of 20 μl at 42°C for 60 minutes. Then, 2 μl of the reverse-transcribed mixture was subjected to PCR [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 20 mM each dNTP (A,G,T,C), 0.5 U Taq polymerase (Takara Bio, Otsu, Japan), and 0.2 5 pmol of each primer in a total volume of 20 μl]. The primer sequences used for the current experiments were given as follows: 5′-TCAACAACCACCTGTTGTGG-3′ as an upstream primer and 5′-TCACCTCAACGATATGCAGC-3′ as a downstream primer for *T-bet*, which yield a 405-bp fragment [23]; 5′-AATGAACGGACAGAACCGG-3′ as an upstream primer and 5′-GGTCCAGATTCAGTGGTTGG-3′ as a downstream primer for *GATA-3*, which yield a 651-bp fragment [23]; 5′-ATGCCTCCTCTTCCTTGA-3′ as an upstream primer and 5′-ATTGTGCCCTGCCCTTCTCA-3′ as a downstream primer for *Foxp3*, which yield a 314-bp fragment [24]; 5′-CGCCTGCGGATTCTACTG-3′ as an upstream primer and 5′-GCAGCTCGAAGAGGCAGT-3′ as a downstream primer for *SOCS1*, which yield a 445-bp fragment [25]; 5′-ATGGTCACCCACAGCAAGTTT-3′ as an upstream primer and 5′-TCACACTGGATGCGCAGGTTC-3′ as a downstream primer for *SOCS3*, which yield a 293-bp fragment [25]; and 5′-GAAATCCCAGCACCATCTCCAGG-3′ as an upstream primer and 5′-GTGGTGGACCTCATGGCCCACCATG-3′ as a downstream primer for *GAPDH*, which yield a 781-bp fragment [26]. We used 25, 27, or 30 PCR cycles to amplify the fragments, with each cycle consisting of 94°C for 60 seconds, 55°C for 90 seconds, and 72°C for 150 seconds, with an initial denaturation step of 94°C for 5 minutes and a final

elongation step of 72°C for 5 minutes. PCR was carried out in a DNA Thermal Cycler (Takara Bio). Amplified cDNA was electrophoresed through 1.5% agarose gels containing 100 ng/ml ethidium bromide. After electrophoresis, gels were illuminated with UV light, viewed, and photographed (Polaroid type 667 film; Polaroid Corp, Cambridge, MA). Densitometric analysis for the RT-PCR band patterns was done using NIH Image 1.59 software (National Institutes of Health, Bethesda, MD). The relative density of each specific RT-PCR band was expressed as a ratio to the density of GAPDH. The identification of each amplified product was confirmed by automated DNA sequencing.

To determine the optimal condition for the semiquantitative RT-PCR described above, we reverse-transcribed 0.1, 0.5, 1, and 5 μg of total RNA, using identical PCR cycles (from 18 to 40). We decided to use 1 μg of total RNA as a template for RT and to use 25 cycles of PCR amplification for *GAPDH* and *SOCS3*, 27 cycles for *T-bet*, *GATA-3*, and *Foxp3*, and 30 cycles for *SOCS1*.

Induction and Stimulation of DCs

To generate DCs, healthy donor-derived PBMCs (9×10^6 per 3 ml/well) were placed into six-well plastic tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. After 2 hours of incubation at 37°C, nonadherent cells were removed and adherent cells were stimulated with granulocyte-macrophage colony-stimulating factor (500 U/ml; PeproTech, London, United Kingdom) and IL-4 (250 U/ml; Genzyme, Boston, MA) to generate immature DCs [16,19]. Flow cytometric analysis by using each specific antibody revealed that the population of adherent cells remaining in the wells was composed of >95% $CD14^+$ monocytes and that the monocyte-derived cells treated with granulocyte-macrophage colony-stimulating factor and IL-4 were consistently >92% $CD14^-$ $CD1a^+$ human DCs [16]. These DCs were treated with X-ray, 5-FU, and OK-432 as described above. The supernatants were collected for cytokine assay. The cells were provided for allogeneic mixed lymphocyte reaction (MLR).

Allogeneic MLR

To evaluate the antigen-presenting ability of the DCs, we performed an allogeneic MLR test. Different numbers of irradiated (30 Gy) DCs were cultured with 2×10^5 allogeneic T cells (>95% $CD3^+$) purified from PBMCs by anti–CD3-coated microbeads and a magnetic cell sorting system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After 5 days of cultivation, [^{3}H]-thymidine (1 μCi/well) was added to the cultures. Eighteen hours later, [^{3}H]-thymidine uptake was measured using a liquid scintillation counter. Cell-free supernatants were harvested and stored at −80°C until the assay for IFN-γ.

Statistical Analysis

Kaplan-Meier curves and log-rank tests were used to assess differences in survival time between the treatment groups. The relationship in clinical responses was statistically evaluated by two-sided Fisher's exact test. In *in vitro* experiments, the data were evaluated using Student's two-tailed *t* test. Values of $P < .05$ were considered statistically significant.

Results

Antitumor Effect of OK-432 in OSCC Patients Receiving RT and UFT

Although the current study is retrospective, and the number of patients in the RT + UFT treatment group was relatively low as compared with that in the RT + UFT + OK-432 group, no statistically significant relationship was observed between these two groups in background of the patients including age ($P = .891$), sex ($P = 1$), stage ($P = .522$), T ($P = .231$), N ($P = .715$), M ($P = 1$), and primary sites ($P = .768$; Table 1).

Of 67 patients who received OK-432 in combination with RT and UFT, 38 patients (56.7%) became histopathologically tumor-free (CR) after the therapy without surgical resection, and 29 (44.3%) showed partial response (PR). In contrast, of 14 patients who received RT and UFT without OK-432, only 2 patients (14.3%) showed CR, and 12 (85.7%) achieved PR after the therapy. A statistically significant relationship in clinical response between patients who received RT + UFT + OK-432 therapy and those who received RT + UFT alone was observed ($P = .0066$; Table 2). These patients were observed for more than 3 years after the therapies. Both overall survival and progression-free survival of the RT + UFT + OK-432 group were statistically significantly longer than in the RT + UFT group ($P = .0075$ and $P = .0175$, respectively; Figure 1*A*). In the current cases, when any residual tumor cells were detected in biopsy material(s), surgical resection of the tumor(s) was performed. However, residual tumors in eight patients were not surgically resected, as described in the Patients and Treatment Protocol section. Thus, in Figure 1*A*, *right graph*, "progression" contains tumor progression, relapse, and appearance of new lesion.

Next, we elaborate on the clinical findings in three patients. In a patient diagnosed with SCC of the lower gingiva with severe bone destruction and invasion, the T4 tumor had completely disappeared by the completion of therapy with RT + UFT + OK-432. Furthermore, new bone formation was observed after the tumor had completely regressed (Figure 1*B*). In a patient with SCC of the tongue, complete remission of the large tongue tumor was observed after the therapy (Figure 1*C*). In a patient diagnosed with SCC of the maxilla with metastasis to cervical lymph nodes, computed tomography demonstrated the disappearance of the metastatic lymph nodes, and the relapse of the tumor has not been observed in more than 6 years since the completion of therapy (Figure 1*D*).

Effect of 5-FU and X-ray Irradiation in OK-432–Induced Cytokine Production and mRNA Expression of Transcription Factors in Human PBMCs: Regulation of Th1/Th2/Regulatory T cell Balance

We have demonstrated that the antitumor effect of RT + UFT therapy was augmented by OK-432. Recently, it has been suggested that certain chemotherapeutic agents, including gemcitabine, 5-FU, a metronomic dose of cyclophosphamide as well as irradiation, might be stimulatory to host immunity or be inhibitory to suppressors of host responses such as regulatory T cells (Tregs) and myeloid-derived suppressor cells and

Table 2. Comparison of Responses to RT + UFT Therapy with and without OK-432.

Clinical Responses	Treatment		
	RT + UFT + OK-432 (%)	RT + UFT (%)	Total (%)
CR	38 (56.7)	2 (14.3)	40 (49.4)
PR	29 (44.3)	12 (85.7)	41 (50.6)
SD	0	0	0
PD	0	0	0
Total	67 (100)	14 (100)	81 (100)

The statistical relationship was detected by two-sided Fisher extract test ($P = .0066$).

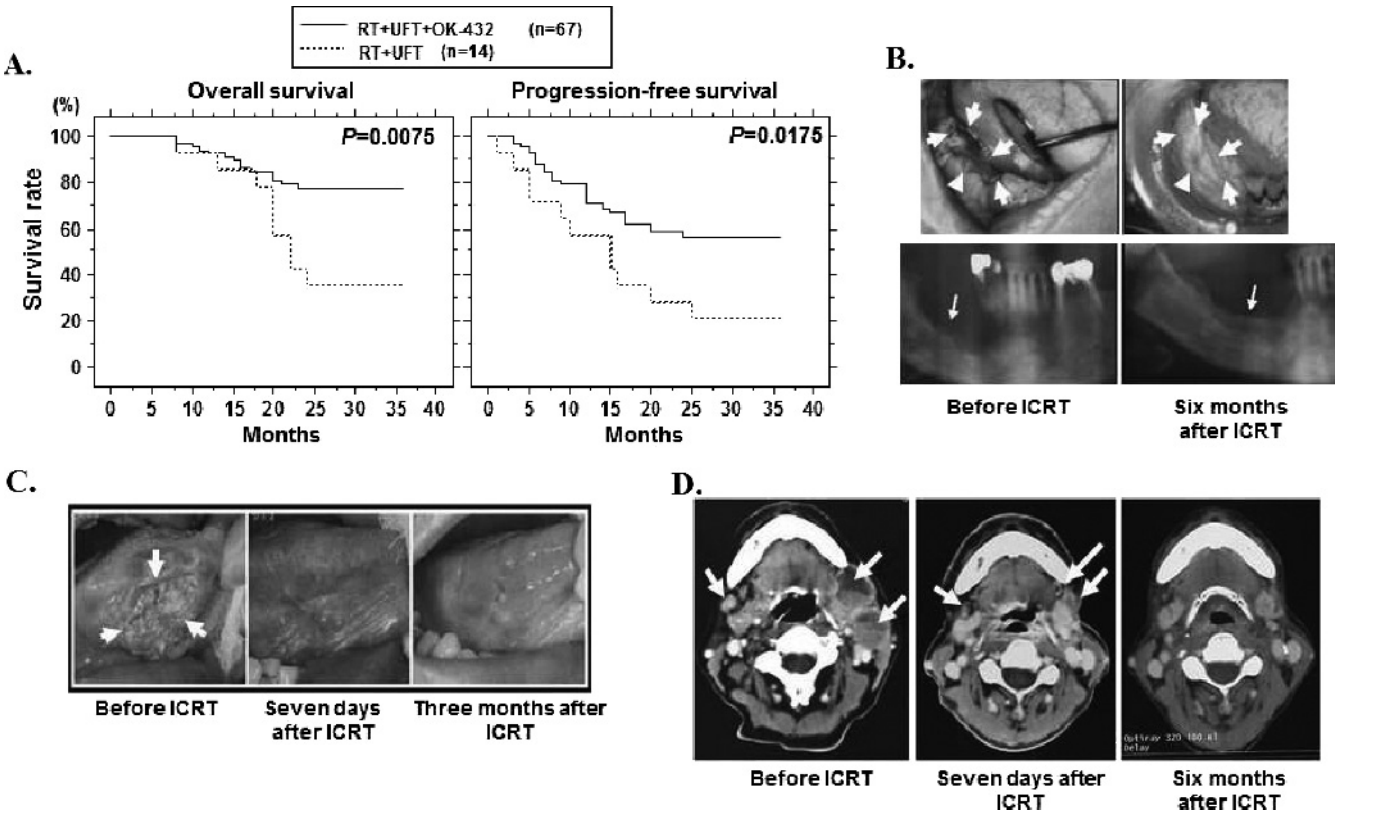

Figure 1. Antitumor effect of OK-432 in combination with RT and UFT. (A) Overall survival (left graph) and progression-free survival (right graph) of patients who received RT + UFT + OK-432 therapy (solid lines) or RT + UFT therapy (dotted lines). (B) SCC of the lower gingiva with severe bone destruction and invasion. The T4 tumor indicated by arrows in the upper left photograph had completely disappeared after RT + UFT + OK-432 therapy, as indicated by arrows in the upper right photograph. The arrow in the lower left photograph indicates bone destruction, and the arrow in the lower right photograph indicates new bone formation. (C) SCC of the tongue. Arrows in the left photograph indicate the primary tumor. (D) SCC of the maxilla with metastasis to cervical lymph nodes. Pictures show computed tomography of metastatic cervical lymph nodes. Arrows in the left and center pictures indicate metastatic lymph nodes.

therefore enhance the effect of cancer immunotherapy [27–29]. Thus, we next conducted *in vitro* experiments to examine whether 5-FU and radiation may augment antitumor host responses induced by OK-432. First, we investigated the effect of 5-FU and X-ray irradiation in OK-432–induced cytokine production. $CD4^+$ helper T cells that are major cytokine-producing cells are divided into two subpopulations, Th1 and Th2 cells. Th1 cells are induced by IL-12 or IL-18 and produce IFN-γ, IL-2, and TNF-α. Th2 cells are induced by IL-4 and produce IL-4, IL-5, IL-6, IL-10, and IL-13 [30]. Th1-type cytokines may be effective in eliminating cancer cells, and Th2-type cytokines may inhibit a Th1-mediated antitumor effect [31]. Furthermore, recent reports suggest that $CD4^+CD25^+Foxp3^+$Tregs secrete TGF-β and IL-10 and inhibit antitumor immunity [32]. It is important to shift the cytokine balance to Th1 to obtain a therapeutic benefit in cancer therapy.

Healthy donor-derived human PBMCs were treated with OK-432, 5-FU, and/or X-ray. OK-432 induced all of the cytokines tested. 5-FU slightly augmented OK-432–induced TNF-α production in the PBMCs ($P < .05$). No significant effect of 5-FU in the induction of IFN-γ, IL-12, or IL-18 was observed. X-ray irradiation also elicited no significant change in OK-432–induced Th1-cytokine production. Interesting results were obtained in the production of cytokines inhibitory to antitumor immunity. Both IL-10 and TGF-β were also produced by OK-432–stimulated PBMCs. 5-FU slightly but significantly inhibited OK-432–induced IL-10 production ($P < .05$) and markedly inhibited TGF-β production ($P < .01$). Although IL-10 production was significantly decreased by X-ray irradiation ($P < .01$), no significant inhibition by X-ray in OK-432–induced TGF-β was observed (Figure 2*A*).

Next, PBMCs were treated by 5-FU or X-ray at 0, 3, 6, and 12 hours before adding OK-432. Data were shown in Figure 2*B*. 5-FU slightly augmented OK-432–induced IFN-γ production when 5-FU was added at 3 hours before OK-432 stimulation ($P < .05$). 5-FU exhibited no significant effect on IL-12 production. Likewise, we observed no significant effect of X-ray irradiation on Th1-type cytokine production induced by OK-432. Significant inhibition of IL-10 and TGF-β production induced by OK-432 was observed when PBMCs were stimulated by 5-FU or X-ray at almost all points before adding OK-432 ($P < .01$–$.05$; Figure 2*B*).

T-bet, GATA-3, and Foxp3 are important transcription factors for Th1, Th2, and Tregs, respectively. We next examined the effect of OK-432, 5-FU, and X-ray irradiation on gene expression of these transcription factors and investigated the regulation of Th1/Th2/Treg balance. OK-432 increased the expression of *T-bet* mRNA as well as those of *GATA-3* and *Foxp3*. 5-FU and X-ray elicited no significant change in *T-bet* expression, while markedly decreased the expression of *GATA-3* and *Foxp-3* mRNAs increased by OK-432 (Figure 2*C*).

Although it has been reported that OK-432 is a potent Th1 inducer, several reports have also suggested that OK-432 induces the production of IL-10 and TGF-β mediated by a negative feedback mechanism [12,33]. The findings obtained from the above experiments strongly suggested that 5-FU and X-ray irradiation might enhance the Th1-dominant state induced by OK-432 by inhibiting differentiation of $CD4^+$T cells to Th2 and Tregs, although augmentation of the Th1 cytokine–inducing ability of OK-432 by these agents was only marginal.

Role of SOCS1 and SOCS3 in the Th1-Dominant State Induced by OK-432, 5-FU, and X-ray Irradiation

SOCSs act as negative feedback regulators following immunological stimuli, and there is evidence to suggest that SOCS1 and SOCS3 might be Th1 inhibitors [34,35]. Therefore, we next examined the expression of mRNAs for SOCS1 and SOCS3 in PBMCs stimulated with OK-432, 5-FU, and X-ray. OK-432 increased the expression of mRNAs of SOCS1 and SOCS3. 5-FU and X-ray irradiation inhibited the expression of both genes enhanced by OK-432 (Figure 3*A*). To investigate the role of SOCS1 and SOCS3 in the Th1-dominant state induced by OK-432, we assessed the effect of antisense ODNs for *SOCS1* and for *SOCS3* in OK-432–induced cytokine production. Antisense ODNs for *SOCS1* and *SOCS3* elicited no significant change in the production of IFN-γ or IL-12 induced by OK-432, while these antisense ODNs significantly inhibited OK-432–induced production of IL-10 and TGF-β ($P < .01$–$.05$; Figure 3*B*). These results were similar to those from the experiments using 5-FU and X-ray.

Effect of 5-FU and X-ray Irradiation on the Function of DCs Activated by OK-432

We previously demonstrated that OK-432 induces maturation of DCs and that OK-432–stimulated DCs can induce tumor antigen–specific cytotoxic T lymphocytes *in vitro* as well as *in vivo* [15,16,19]. In the present study, we thus examined the effect of 5-FU and X-ray irradiation on DC function activated by OK-432. Although IL-12 production by OK-432–stimulated DCs was slightly increased by X-ray irradiation ($P < .05$), production of TGF-β was significantly decreased by treatment with 5-FU and by X-ray ($P < .01$–$.05$; Figure 4*A*). Other

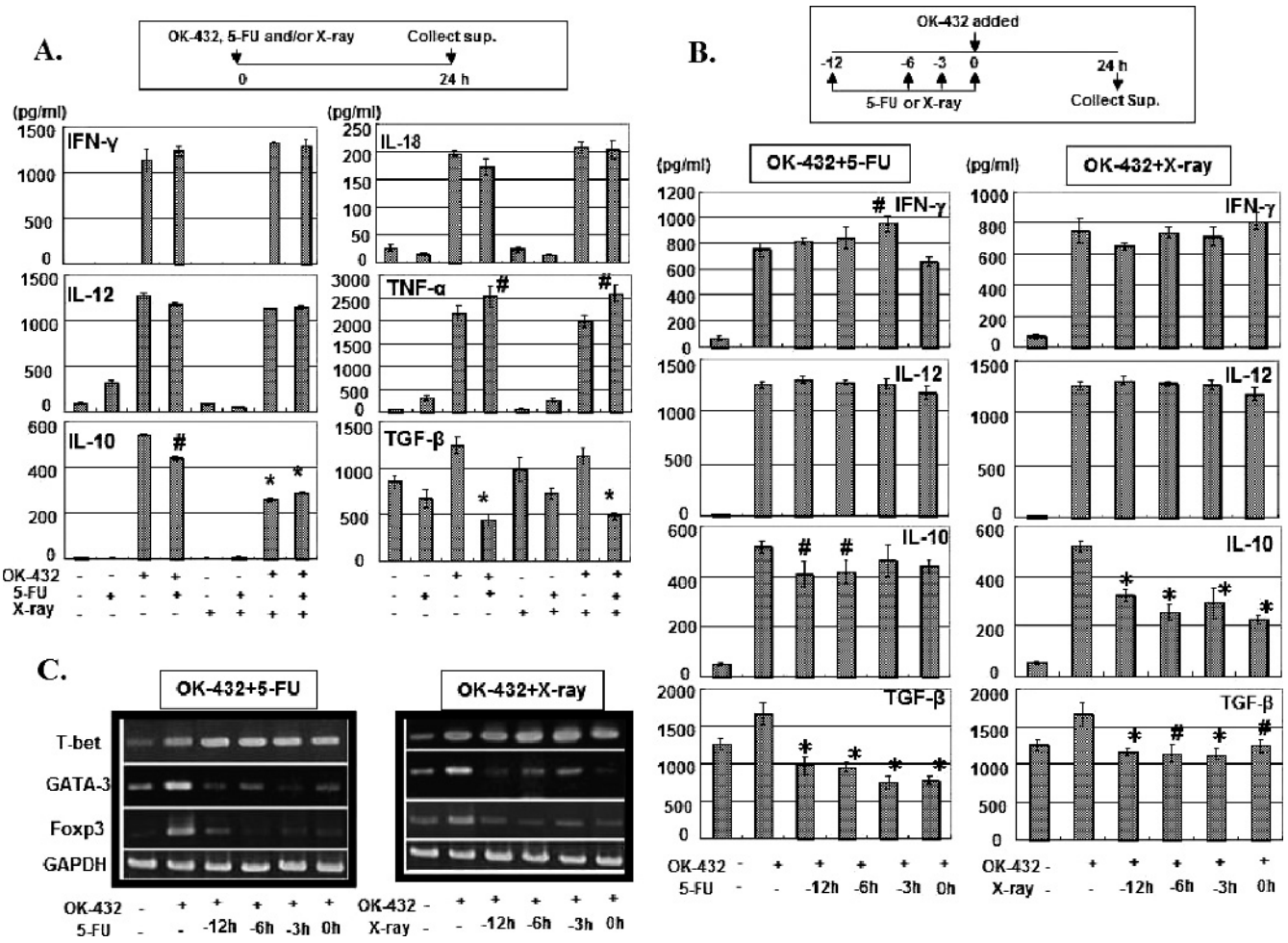

Figure 2. Effect of 5-FU and X-ray irradiation on OK-432–induced cytokine production and mRNA expression of transcription factors in human PBMCs: Regulation of Th1/Th2/Treg balance. (A) Healthy donor-derived PBMCs were treated with OK-432 (1 μg/ml), 5-FU (0.5 μg/ml), and/or X-ray (2 Gy) at 24 hours before collecting supernatants, and then the supernatants were analyzed for cytokines. (B and C) The PBMCs were irradiated (2 Gy) or were treated with 5-FU (0.5 μg/ml) at 0, 3, 6, or 12 hours before OK-432 stimulation. After OK-432 was added, the supernatants were analyzed for cytokines (B), and mRNAs isolated from the PBMCs were assayed for expression of *T-bet*, *GATA-3*, and *Foxp3* (C). $^*P < .01$, $^{\#}P < .05$ as compared with respective controls treated with OK-432 alone.

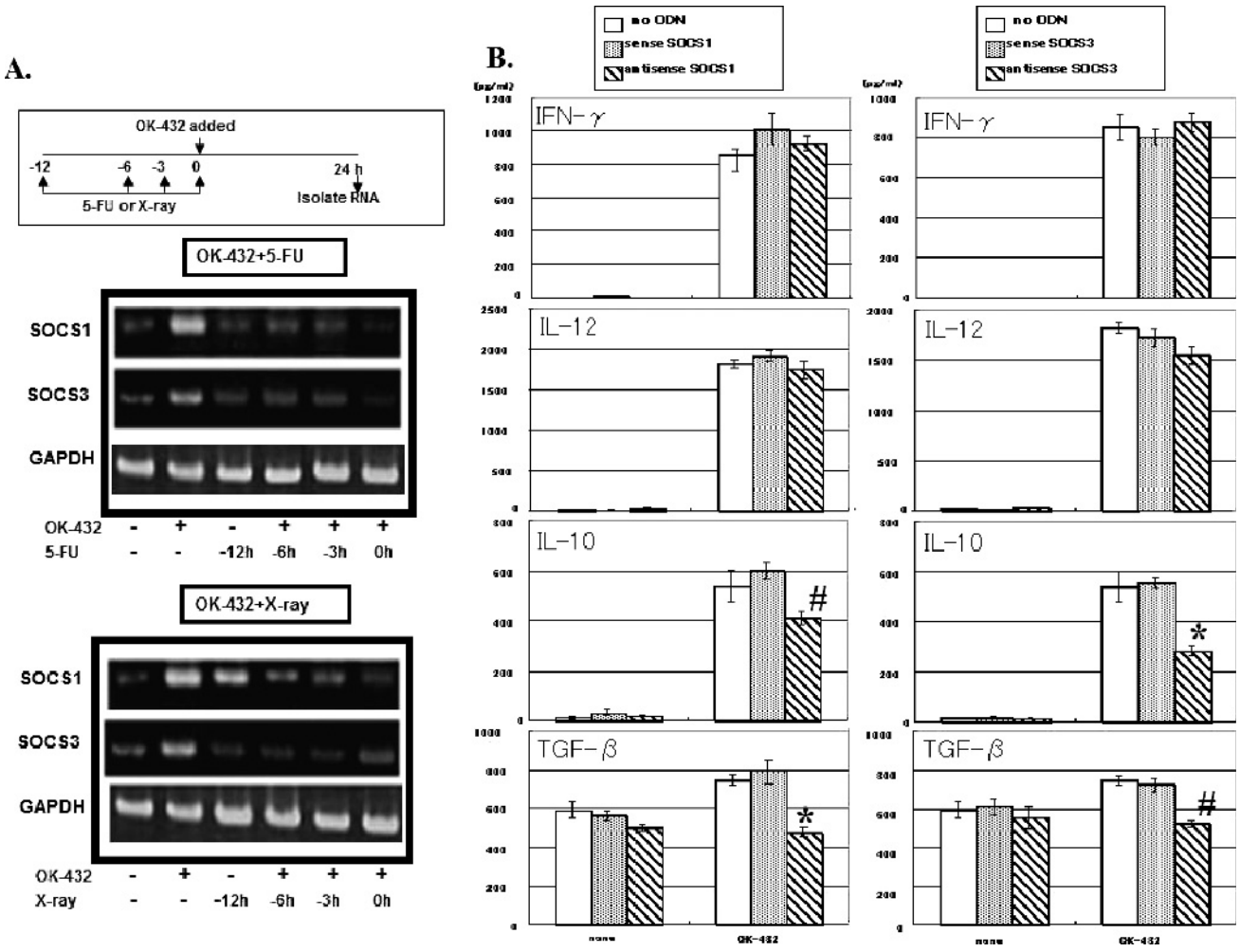

Figure 3. Role of SOCS1 and SOCS3 in the Th1-dominant state induced by OK-432, 5-FU, and X-ray irradiation. (A) Effect of 5-FU and X-ray in OK-432–induced mRNAs for SOCS1 and SOCS3. PBMCs were irradiated (2 Gy) or were treated with 5-FU (0.5 μg/ml) at 0, 3, 6, or 12 hours before OK-432 stimulation (1 μg/ml). After OK-432 was added, we assayed the expression of mRNAs for SOCS1 and SOCS3 in the PBMCs. (B) Effect of antisense ODNs of *SOCS1* and *SOCS3* mRNAs in OK-432–induced cytokines. Sense and antisense ODNs (10 μM) of these molecules were added with Effectene Transfection Reagent into the PBMC culture treated with OK-432. After 24 hours, the supernatants were analyzed for cytokines. $^{*}P < 0.01$, $^{\#}P < 0.05$ as compared with respective controls treated with OK-432 alone.

cytokines tested were not detected in supernatants derived from any of the DC cultures (data not shown). To evaluate the antigen-presenting ability of DCs, we performed an allogeneic MLR test in which DCs treated with each stimulus were co-cultured with allogeneic T cells. In comparison with DCs treated with OK-432 alone, OK-432–treated DCs co-stimulated with 5-FU and/or X-ray showed a higher ability to stimulate the proliferation of allogeneic T cells (data not shown). The cell-free supernatants derived from the allogeneic MLR culture were analyzed for IFN-γ. Data from the allogeneic MRL culture at a DC/T ratio of 1:20 are shown in Figure 4*B*. Similar to the data obtained from the proliferation assay, allogeneic T cells co-cultured with DCs stimulated by OK-432 in combination with 5-FU and/or X-ray secreted IFN-γ more than did those co-cultured with DCs treated with OK-432 alone ($P < .01$–$.05$). DCs stimulated with any stimuli did not produce IFN-γ.

Discussion

Several investigators including our group demonstrated that irradiation and treatment with certain chemotherapeutic agents might augment host immunity and reported the data suggesting the possibility that immunotherapy in combination with RT and/or chemotherapy may be effective in patients with malignancies [27–29,36,37], while there are only a few reports that clearly showed that ICRT practically prolonged the survival of patients with malignant diseases including OSCC, as compared with CRT alone. The results obtained from the current study clearly indicated that the combination therapy by using RT, UFT, and OK-432 had a striking anticancer effect far better than that with RT + UFT alone and strongly suggested that the ICRT might be efficient in the treatment of OSCC patients. Although the current study is retrospective, and the number of patients in the RT + UFT treatment group was relatively low as compared with that in the RT + UFT + OK-432 group, our study results might be beneficial for establishing more effective cancer therapy, as a statistically significant difference was still obtained.

Although these findings were obtained using the nonspecific immunotherapeutic agent OK-432, recently called an "old biological response modifier (BRM)," we expect CRT to enhance the antigen-specific immunity induced by specific immunotherapy such as cancer vaccines,

because we have already reported that one of the principal effects of OK-432 is enhancement of antigen-specific immunity through maturation of DCs mediated by TLR4 signaling [9,15,16]. We have already reported that the cancer vaccine based on OK-432–treated DCs in combination with gemcitabine and/or S-1 might prolong the survival of the patients with advanced pancreatic carcinoma refractory to standard treatment in a retrospective clinical study [38], and further, we have started a prospective clinical trial of a DC-based cancer vaccine in combination with chemotherapy in several types of malignancies. We expect the therapy to demonstrate an anticancer ability.

CRT is used as a first-line treatment for many inoperable cases as well as for operable cases as an adjuvant or a neoadjuvant therapy, and the present findings strongly suggest that ICRT may be available as a first-line therapy for many types of malignancies. Previously, in most cases, cancer patients have received immunotherapies after the failure of standard treatments, since it was then believed that the combination of standard CRT and immunotherapy was not favorable because of inhibitory effects of host immunity by the standard CRT. However, many investigators and clinicians now understand that taking the immunotherapy to patients at an earlier stage is more effective for cancer treatment. We expect the antitumor effect of ICRT as a first-line therapy. In addition, recently a lot of molecular targeting agents such as tyrosine-kinase inhibitors, which have little inhibitory effects as well as having stimulating ability in host immunity [39], are being developed to be used clinically. There is also great interest in the combination therapy using tyrosine-kinase inhibitors as well as metronomic chemotherapy and cancer vaccines. We are now also planning a clinical trial using this combination.

Th1 shift in cytokine balance is an important event in this therapy combined with an immune adjuvant OK-432. We have reported that the patients whose serum IFN-γ levels were increased after OK-432 administration showed favorable clinical outcome [9,40]. In the present findings, although augmentation of IFN-γ secretion by 5-FU and irradiation was detected only little in the experiments using PBMCs (Figure 2*B*), allogeneic MLR using DCs treated with OK-432, 5-FU, and/or X-ray demonstrated IFN-γ production significantly more than that using DCs stimulated only by OK-432 (Figure 4*B*). DCs may play significant roles in the oral cancer patients who received OK-432.

The findings from the present *in vitro* experiments clearly demonstrated that X-ray irradiation and 5-FU augmented Th1 responses induced by OK-432 through regulating IL-10 and TGF-β mediated by the inhibition of negative regulators SOCS1 and SOCS3. Although OK-432 induces IL-12 and IFN-γ as potent Th1 inducers, IL-10 is also induced through a negative feedback loop at 12 to 24 hours after OK-432 stimulation [[12] and authors' personal data]. It was also reported that TGF-β is induced by OK-432 [33]. In the present *in vitro* experiments, expression of mRNAs for SOCS1 and SOCS-3 was increased at least within 24 hours after adding OK-432 into the PBMC

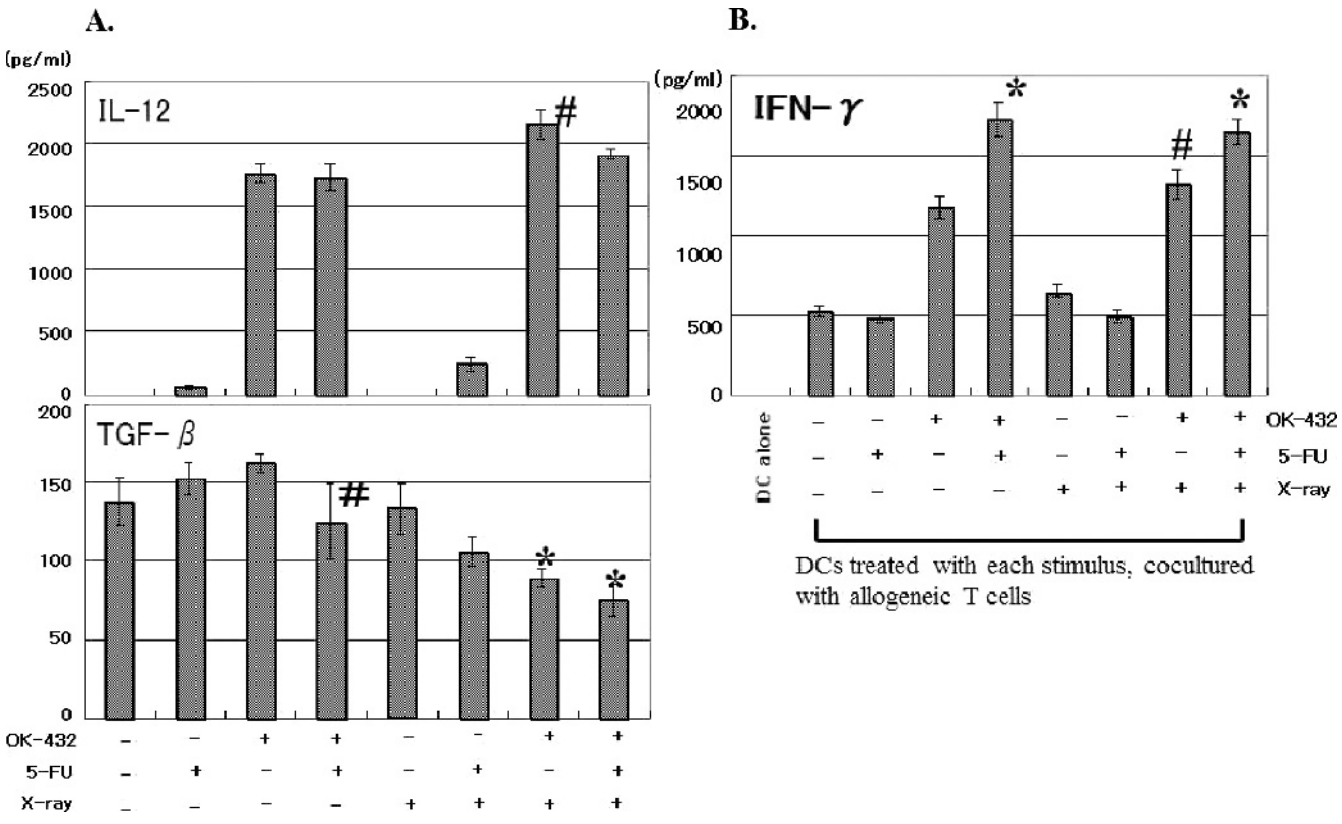

Figure 4. Effect of 5-FU and X-ray irradiation on the function of DCs activated by OK-432. (A) Production of IL-12 and TGF-β by DCs treated with OK-432, 5-FU, and X-ray. Monocyte-derived DCs were treated with OK-432 (1 μg/ml), 5-FU (0.5 μg/ml), and/or X-ray (2 Gy) at 24 hours before collecting supernatants, and then the supernatants were analyzed for IL-12 and TGF-β. (B) IFN-γ production by allogeneic T cells stimulated by DCs treated with OK-432, 5-FU, and X-ray. Different numbers of irradiated (30 Gy) DCs were cultured with 2×10^5 allogeneic T cells (>95% $CD3^+$) purified from PBMCs by anti–CD3-coated microbeads and a magnetic cell sorting system. After 5 days of cultivation, cell-free supernatants were analyzed for IFN-γ. Data from the experiments performed at a DC/T ratio of 1:20 are shown. $^*P < 0.01$, $^{\#}P < 0.05$ as compared with respective controls treated with OK-432 alone.

culture. It was reported that SOCS3 inhibits Th1 cells by suppressing IL-12–mediated signal transducer and activator of transcription 4 and that SOCS3 regulates the onset and maintenance of Th2-mediated allergic response [34,35]. Furthermore, SOCS1 completely inhibits TLR4 signaling, and SOCS3 blocks the TLR4-mediated IL-6 signal but not the IL-10 signal [34,41], while OK-432 augments antitumor immunity through TLR4 signaling, as reported by our group [9,15,16,19]. Thus, it appears that 5-FU and X-ray irradiation might interrupt the negative feedback loop by inhibiting expression of *SOCS1* and *SOCS3* genes and then augmenting the OK-432–induced Th1 response in patients with OSCC. In the future, molecular targeting therapy against SOCSs in combination with immunotherapy may be useful in the treatment of patients with malignancies. The development of new molecular targeting is ongoing in our laboratory by using small interfering RNA technology.

On the basis of the findings of the current study, we have proposed the concept of a new cancer therapy called ICRT as well as new therapeutic targets that are negative regulators for antitumor host immunity.

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